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Original article

The reduction of IL-6 gene expression, pAKT, pERK1/2, pSTAT3 signaling pathways and invasion activity by gallic acid in prostate cancer PC3 cells

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ABSTRACT

Prostate cancer (PC) is one of the most common cancers among men. Progression of prostate cancer is associated with an increase in cellular level of interleukin-6 (IL-6). Gallic acid (GA) is a polyhydroxy phenolic compound which can inhibit the growth of cancer cells. The aim of this study was to evaluate the effects of GA treatment on cell viability, proliferation, invasion, IL-6 gene expression, IL-6 secretion, cellular levels of pSTAT3, pERK1/2, and pAKT signaling proteins in human prostate cancer PC3 cells. PC3 cells viability after treatment with GA (0–120 μM) was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The expression of *IL-6* was investigated using real-time polymerase chain reaction. Cellular concentration of pSTAT3, pERK1/2, and pAKT signaling proteins were determined by Western blotting technic. PC3 cells invasion was assessed by invasion assay test. Treatment with GA caused a significant decrease in cell viability, proliferation, invasion, cellular levels of pSTAT3, pERK1/2, and pAKT signaling proteins after 48 h in a dose-dependent manner. The level of IL-6 and its gene expression decreased significantly in PC3 cells treated with GA. Our results show that IL-6 down-regulation and decreased IL-6 protein level in PC3 cells by GA resulted in diminishing of pSTAT3, pERK1/2, and pAKT signaling proteins which lead to the reduction of the cell survival, proliferation, and invasion in PC3 cells. Therefore, it seems that GA can be considered an anticancer agent in the treatment of prostate cancer.

1. Introduction

Prostate cancer (PC), the most frequently diagnosed non-cutaneous cancer, is one of the most common cancers among men which is androgen dependent in initial stage. PC, however, eventually progresses to be androgen-independent phenotype [1]. Studies show that PC in the form of androgen-independent is associated with a significant increase in IL-6 [2]. IL-6 is a multi-functional cytokine that plays a role in numerous physiological processes, including growth and immune responses. It can also be involved in many malignant tumors such as myeloma, renal cell carcinoma, cervical carcinoma and prostatic carcinoma [3]. IL-6 not only has a key role in the transition of PC from an androgen-dependent to an androgen-independent state but also it plays an important role in prostate cancer progression through a number of signaling pathways such as JAK-STAT, PI3k-AKT, MAPk-ERK, and especially JAK-STAT pathway [4–6]. Therefore, investigations on IL-6 signaling pathways can be effective in achieving new therapeutic approaches in oncology.

Studies have shown that consumption of fruit and vegetables in the diet can reduce the risk of different diseases especially some cancers [7–9]. In the past decade, several researches have been done to exam the role some antioxidants as natural anti-tumor products in prostate cancer cell lines [10,11]. Gallic acid (GA) is a polyhydroxy phenolic compound that is widely found in grapes vegetables and many fruit. GA has anti-inflammatory, anti-virus, and free radical scavenger activities [12]. Also, it is reported that GA can inhibit growth of cancer cells by inducing apoptosis through the ROS-mediated pathways in human prostate cancer cells [13]. Therefore, the aim of this study was to investigate the effects of GA on the IL-6

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gene expression, pSTAT3, pAKT, pERK1/2 cellular signaling proteins, and invasion in human prostate cancer PC3 cells.

2. Experimental procedures

2.1. Chemicals and antibodies

The human PC3 cell line was obtained from Pasteur Institute of Iran (Tehran, Iran). Gallic acid, dimethyl sulfoxide (DMSO), matrigel (ESH Sarcoma Matrigel), and 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) were purchased from sigma (St. Louis, MO, USA). RPMI 1640 medium, trypsin 0.25% and fetal bovine serum were obtained from Gibco (Rockville, MD, USA). SYBR Green PCR Master Mix was obtained from Qiagen Co. (Düsseldorf, Germany). Polyvinylidene difluoride (PVDF) membrane and BM Blue POD Substrate Precipitating were prepared from Roche. IL-6 ELISA kit was obtained from AviBion. BIOZOL Total RNA Extraction kit was prepared from Bio Flux. Antibodies were purchased from Abcam (San Francisco, CA, USA). All other chemicals used were of analytical grade.

2.2. Cell culture and cell viability assay

PC3 cells were cultured in RPMI1640 medium enriched with L-glutamine, 10% fetal bovine serum (FBS), 10^2 U/ml penicillin and 0.1 mg/ml streptomycin under standard conditions (37 °C, 95% humidity and 5% CO_2). The viability and proliferation of PC3 cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide test (MTT assay test) [14]. Briefly, PC3 cells were seeded in 96-well plates at a density of 5000 cells/well in RPMI 1640 medium containing 10% FBS and plates were incubated for 24 h. The medium was replaced with medium containing different concentrations of GA (0–120 μM) and incubated for 48 h. Then, 100 ml of colorless RPMI 1640 and 10 μl of MTT 12 mM were added to each well and incubated for 4 h. The medium was removed and 50 μl of DMSO was added to each well and incubated for 10 min in order to dissolve the formazan crystals. This test was done in triplicate for each concentration of GA. Viability percentage was calculated after reading absorbance of formazan at 490 nm using a microplate reader (Stat Fax 3200, Awareness Technology, USA) based on the following formula: Viability = A (sample)/A (control) × 100. Moreover, non-treated cell viability was set as 100%.

2.3. *Real-time polymerase chain reaction (RT-PCR) for IL-6*

PC3 cells were incubated with 0, 25, 30, and 35 μM of GA for 48 h. Then, total mRNA was extracted for each well by Biozol reagent according to the manufacturer's protocol. RT-PCR for *IL-6* gene expression was done using Rotor-Gene 3000 (Corbett, Australia) as described previously [10]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an endogenous control gene for the normalization of *IL-6* expression.

2.4. Western blot analysis

Treated PC3 cells with different concentrations of GA (0, 25, 30, and 35 μM) were harvested after 48 h. Western blot analysis for pSTAT3, pAKT, and pERK1/2 cellular signaling proteins were carried out as described previously [10]. β-actin assessed as an internal control.

2.5. Measurement of IL-6

PC3 cells were treated with various concentrations of GA (0, 25, 30, and 35 μM) in 6-well plates for 48 h. Then, their media were collected to measure the levels of IL-6 using an ELISA kit (AViBion Human IL-6 ELISA kit). All steps of this procedure were performed according to the manufacturer's protocols. Finally, the optical density was measured at 450 nm using an ELISA reader (Stat Fax® 3200 Microplate Reader, Awareness Technology, USA).

2.6. Invasion assay

Transwells with 8 μm-pores, 24-well plates, and matrigel were used for invasion assay. The upper chamber of the transwell was coated with matrigel at a concentration of 1 mg/ml and the lower chamber was filled with 10% FBS-medium as chemoattractan. PC3 cells were treated with different concentrations of GA (0, 25, and 35 μM) in 24 well plates for 48 h. Then, the cells were detached by trypsin and were resuspended in 0.1% FBS-medium. PC3 cells (5×10^4 per well) were added into the upper chamber of the transwell and were incubated at 37 °C in 5% CO2 for 24 h. Subsequently, cells on the upper chamber of the transwell were scraped with a cotton swab. Cells on the bottom side of the transwell (migrated cells) were fixed by 5% glutaraldehyde and were stained with 0.5% toluidine blue solution. Finally, the fixed cells in the low chamber of the transwell were counted using inverted microscope. Three independent areas per well were counted and the mean number of migrated cells was calculated [15].

2.7. Statistical analysis

The results were expressed as mean ± SD. All statistical analysis were performed using SPSS version 20.0 software (SPSS, Chicago, IL, USA) and prism 5 software. Group means were compared by Kruskal-Wallis test for multiple comparisons and $P < 0.05$ was considered statistically significant. Inhibitory concentration of 50% (IC50) was calculated by the Probit procedure (SPSS software, version 20). Analysis of relative gene expression data was estimated with the ΔΔCT method and the data were expressed as fold change. Melting curves were generated to ensure the purity of the amplification product of each reaction.

3. Results

3.1. The effect of GA on PC3 cells proliferation

Fig. 1 shows the effect of GA in different concentrations range (0–120 μM) on PC3 cells proliferation after 48 h. Results showed

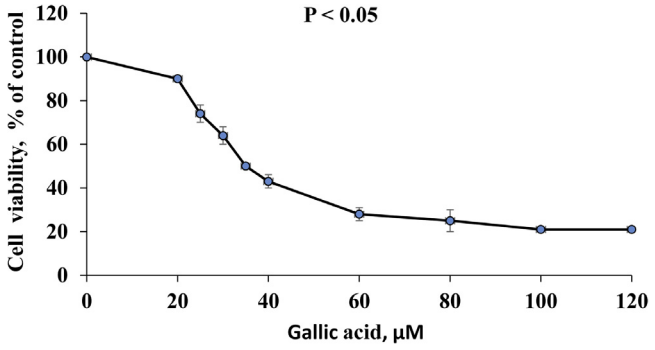

Fig. 1. The inhibition of PC3 cells proliferation by GA. PC3 cells were seeded in 96-well plates for overnight and were then treated with 0–120 μM gallic acid in DMSO or DMSO alone for 48 h. At the end of treatment time, cell viability was measured by MTT assay. Data indicate mean ± SD, n = 3.

that the survival and proliferation of PC3 cells were significantly decreased ($P < 0.05$) by increasing concentration of GA. The IC_{50} calculated for GA was 50 μM on PC3 cells. Also, treatment by GA resulted in morphological changes in PC3 cells (Fig. 2).

3.2. The effect of GA on IL-6 gene expression in PC3 cells

Fig. 3 shows the effect of GA on the gene expression of *IL-6* in PC3 cells. *IL-6* gene expression in treated PC3 cells with GA significantly down-regulated ($P < 0.05$) in a dose-dependent manner. In treated PC3 cells with GA, reduction of *IL-6* expression was significantly declined by 51.05, 85.87 and 99.72 fold at 25, 30, and 35 μM compared to untreated cells, respectively.

3.3. Effect of GA on IL-6 protein secretion

Fig. 4 shows that treatment of PC3 cells with different concentrations of GA resulted in a significant reduction ($p > 0.05$) in secretion of IL-6 (25.85%, 46.23% and 60.16% at 25, 30 and 35 μM of GA, respectively) when compared with control cells.

3.4. Effects of GA on the cellular signaling pathways in PC3 cells

Fig. 5 shows the effect of GA on the cellular levels of phosphorylated STAT3, ERK1/2, and AKT signaling proteins. GA partially reduced the levels of pSTAT3 and pAKT at a dose of 25 μM. On the other hand, a significant reduction in the levels of pSTAT3 and pAKT was seen after treatment by GA at 30 and 35 μM. Reduction in cellular level of pERK1/2 was much lower than that of pAKT and pSTAT3 at a dose of 35 μM.

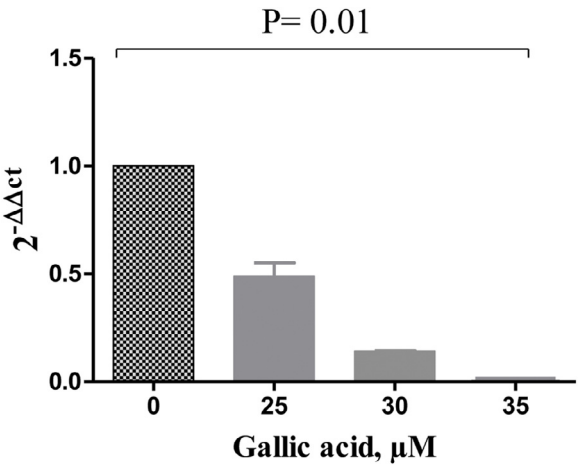

Fig. 3. The effect of GA on *IL-6* gene expression. The expression of *IL-6* was significantly down-regulated in PC3 cells treated with different concentrations of GA after 48 h. Columns and bars represent mean ± SD of three independent experiments. The expression of *IL-6* normalized with glyceraldehyde-3-phosphate dehydrogenase as an internal standard.

3.5. Effects of GA on PC3 cells invasion

PC3 cells potential invasion was significantly reduced after treatment with GA in a dose-dependent pattern (Fig. 6). Treatment by GA at 25 and 35 μM significantly reduced PC3 cell invasion by 48.8% and 64.9% compared to control cells, respectively.

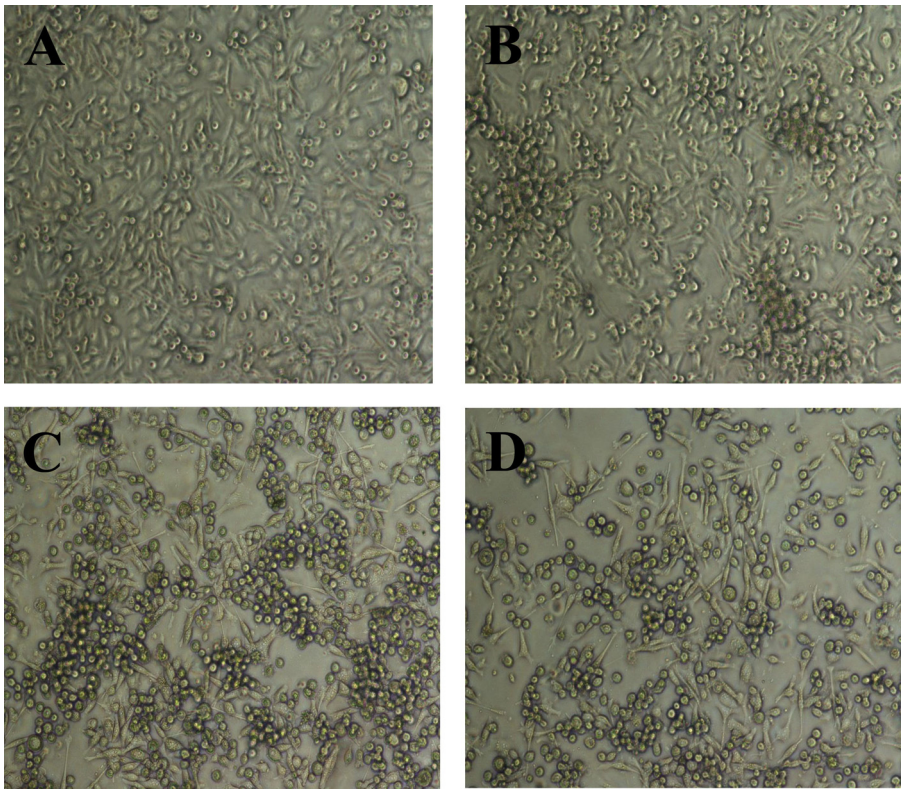

Fig. 2. Morphological changes of PC3 cells after treatment with various concentrations of gallic acid (A: 0; B: 25; C: 30; and D: 35 μM, respectively) for 48 h.

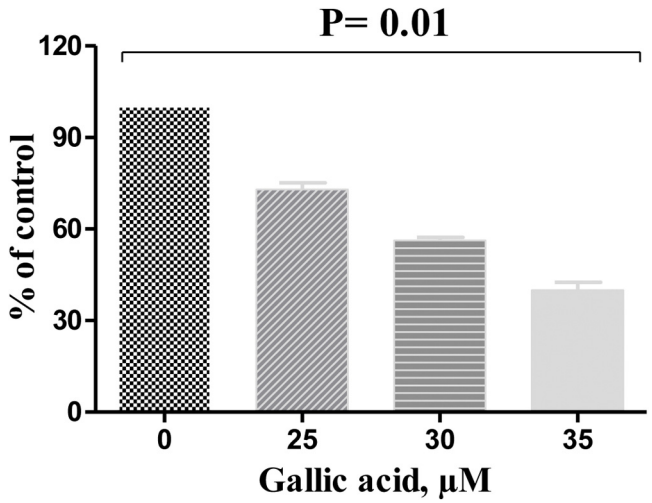

Fig. 4. The levels of IL-6 after treatment with GA. PC3 cells were treated with different concentrations of GA for 48 h and secreted IL-6 was measured. Data represent the mean ± SD value of three consecutive experiments.

4. Discussion

Nowadays, considering the side effects of chemotherapy multiple studies have focused on the therapeutic potentials of antioxidants and plant-derived compounds in the treatment of diseases, especially cancer in recent years [16–18]. The prostate cancer is known as one of the most dangerous malignant diseases in men all over the world [1]. Our results in this study showed that GA resulted in a reduction of cell viability and it caused changes in morphology of PC3 cells (Figs. 1 and 2). Furthermore, GA exhibited anti-proliferative effects on PC3 cells. These results are consistent with previous studies [19,20]. Previous researches have demonstrated that GA has selective toxic effects on human prostate cancer PC3 cell line. These results suggest that tumor growth suppression by GA may partly be attributed to induction of apoptosis in these cells as reported before [21]. Also, previous studies have shown that GA is an anti-cancer agent with cytotoxic effects against prostate cancer DU145 cells [21]. Previous studies showed that antioxidants such as andrographolide can decrease viability, survival, and metastasis in PC3 cells by inhibiting the synthesis of IL-6 [11] which is consistent with the results of our study. Therefore, in this study reduced IL-6 protein secretion by GA (Fig. 4) can contribute to the reduction of viability, survival and invasion in PC3 cells. RT-PCR has also shown a reduction in *IL-6* gene expression (Fig. 3). In previous studies anti-inflammatory and anti-allergic effects of GA were investigated on human basophil cells (cell line KU812). The results suggested that GA can impose these effects through inhibition of the expression of many cytokines such as NF-$_K$B and IL-6 [22]. Also, GA had inhibitory effects on inflammatory mediators such as TNF-α and IL-6 in 93RS2 sertoli cells [23]. In our study, treatment with GA led to a significant reduction in the level of pAKT protein in PC3 cells (Fig. 5). Previous researches have indicated that the phosphorylated form of AKT protein increases in many types of tumors. PI3 K/AKT plays an important role in the progression of prostate cancer by increasing cell proliferation and suppressing apoptosis [24,25]. On the other hand, it was found that GA has anti-metastasis effects on gastric cancer cells through down-egulation of PI3 K/AKT signaling protein [26]. Also, GA can induce apoptosis and inhibit inflammatory cytokines such as IL-6 in fibroblast-like synoviocytes (FLS) in patients with rheumatoid arthritis [27]. Thereby, in this study reduction of pAKT, at least in part, can be a reason for the reduction of viability and proliferation of PC3 cells. Also, based on our results, cellular pSTAT3 signaling protein was diminished by GA (Fig. 5) which is in line with other studies [11]. Published studies have shown that pSTAT3 inhibited apoptosis and resulted in cell survival by up-regulating P53 and increasing of pro-apoptotic factors such as Bcl-2 and Bcl-XL in cancer cells [28]. Therefore, the reduction of pSTAT3 in treated cells with GA that was observed in our study, at least in part, led to declined cells survival in PC3 cells. On the other hand, in our study treatment

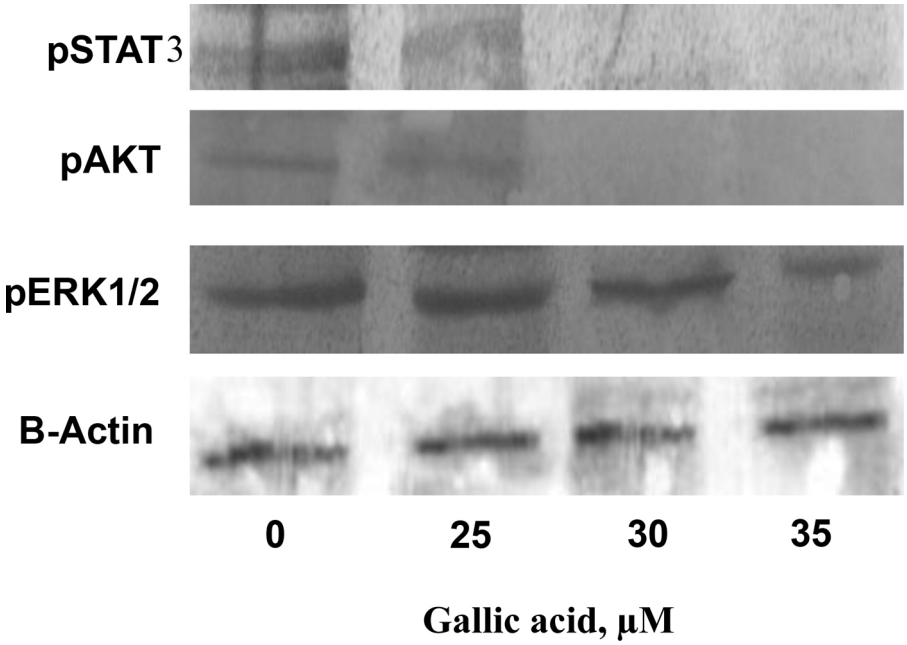

Fig. 5. The effects of GA on the levels of signaling proteins in PC3 cells that were treated with increasing doses of GA for 48 h. The cell lysates were collected and subjected to Western blot analysis. GA down-regulated the expression of pSTAT3, pAKT, and pERK1/2. Equal amounts of lysate protein were subjected to gel electrophoresis.

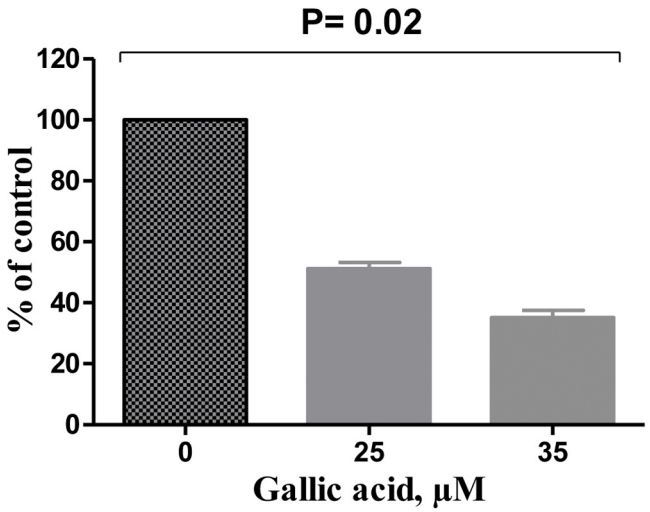

Fig. 6. The effect of GA on the invasion of PC3 cells. Treated PC3 cells with different concentrations of GA for 48 h were placed in invasion chambers for 24 h at 37 °C. Invading cells were counted using inverted microscope. Bars represents the mean ± SD of triplicate determinations.

with GA also reduced cellular level of pERK1/2 protein signaling (Fig. 5). Another study showed that GA can induce apoptosis in HeLa cells by inactivating MAPK/ERK protein signaling pathway [29]. Many studies have shown that MAPK/ERK pathway is frequently activated in prostate cancer. It has been found that IL-6 mediates growth and survival in human prostate cancer cells by activating both MAPK/ERK and PI3K/AKT signaling pathways [30,31]. Moreover, reduction of survival and growth in human pulmonary fibroblast (HPF) cells through reduction in MAPK activity, was observed after treatment by GA in previous studies [32]. Therefore, it seems that the suppression of pERK1/2 pathway by GA in this study, at least in part, has diminished PC3 cells survival.

In our study, GA inhibited PC3 cells invasion in a dose-dependent manner especially in IC50 (Fig. 6) which is in agreement with other investigators [20,33]. Previous studies have shown that increase in pAKT activity can induce metastasis in prostate cancer cells [24]. However, other studies have reported that GA suppresses cell invasion in both human glioma cells and human cervical cancer cells by inhibiting pERK1/2 and pAKT signaling pathways [20,33] which is in line with the findings in our study. Therefore, inhibition of pERK1/2 and pAKT pathways by GA, at least in part, can be considered as a reason for reducing invasion of PC3 cells.

In this study, we did were not investigate the effect of GA on proapoptotic and anti-apoptotic mediators such as p65, caspase-3, Bcl-2, and Bax levels. These factors can play an important role in cell proliferation, survival, apoptosis, and invasion. Therefore, we suggest that future studies focus on the effects of GA on these factors.

Our results show that IL-6 down-regulation and decreased IL-6 protein level in PC3 cells by GA resulted in diminished pSTAT3, pERK, and pAKT signaling proteins which led to the reduction of the cell survival, proliferation, and invasion in PC3 cells. Therefore, it seems that GA can be considered an anticancer agent in the treatment of prostate cancer.

Declaration of interest

The authors declare that there is no conflict of interest.

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